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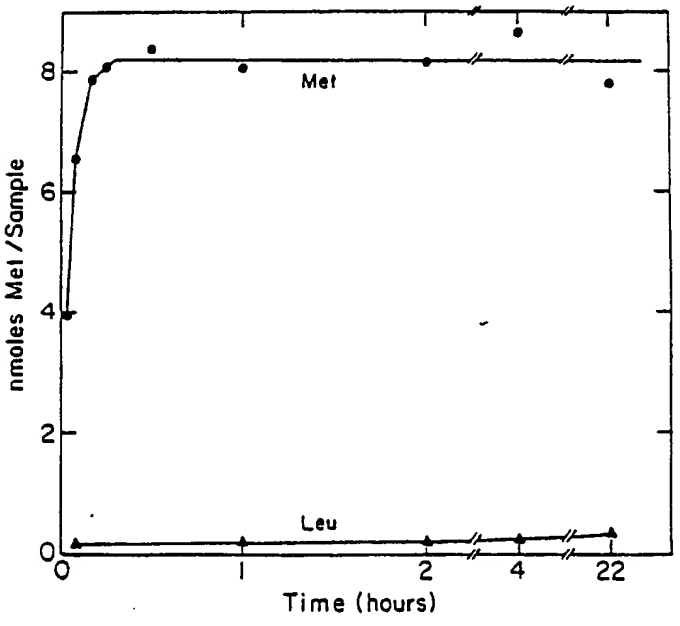
(54) Title: METHOD OF REMOVING N-TERMINAL AMINO ACID RESIDUES FROM EUCARYOTIC POLYPEPTIDE ANALOGS AND POLYPEPTIDES PRODUCED THEREBY

(57) Abstract

A method of sequentially removing one or more N-terminal amino acid residues from an analog of a eucaryotic polypeptide synthesized in a foreign host, comprises contacting the eucaryotic polypeptide analog with aminopeptidase under suitable conditions permitting sequential removal of N-terminal amino acid residues, where the polypeptide analog contains amino acid residue or sequence of residues which blocks the action of an aminopeptidase located at a position other than the N-terminal end of the polypeptide analog. A specific embodiment of the invention concerns removing both an N-terminal methionine residue and its adjacent Leucine residue from a growth hormone analog produced in bacteria by contacting the growth hormone analog with *Aeromonas* aminopeptidase under suitable conditions permitting removal of the N-terminal methionine and Leucine residues. The invention also concerns eucaryotic polypeptide analogs produced in accordance with the methods of the invention.



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METHOD OF REMOVING N-TERMINAL AMINO ACID RESIDUES FROM EUCARYOTIC POLYPEPTIDE ANALOGS AND POLYPEPTIDES PRODUCED THEREBY

Background of the Invention

Recombinant DNA technology permits large scale production of eucaryotic proteins in bacteria. However, the proteins so produced, are frequently characterized by the addition of an extra methionine residue at their N-terminus. This occurs because translation is always initiated at the AUG codon which codes for methionine. In procaryotes, the N-terminal methionine is frequently enzymatically removed. However, it appears that this is not the case for many eucaryotic proteins produced in bacteria. Possibly this is due to the fact that the proteins are massively overproduced and thus overwhelm the bacteria's processing capabilities. Another possible explanation is that the bacterial processing enzymes do not recognize the foreign eucaryotic proteins as their substrates.

In eucaryotes, mature proteins often lack an N-terminal methionine because they have undergone extensive processing by both endopeptidases and exopeptidases, during transport from the site of synthesis to their final location.

As the presence of an N-terminal methionine on eucaryotic proteins may cause an immune reaction when administered to eucaryotes, it would be desirable to process eucaryotic proteins produced in bacteria to remove the N-terminal methionine, thus producing the mature eucaryotic protein. Most available aminopeptidases are zinc metalloenzymes.

They are comprised of several subunits and have very high molecular weights. (For a review see Delange and Smith, The Enzymes, 3rd edition, P.D. Boyer ed., 1971, vol. 3, pp. 81-118). Thus, leucine aminopeptidases from pig kidney and from bovine lens have molecular weights of 255,000 and 320,000, respectively. Although the exact role of these enzymes is not known, it is likely that such high molecular weight enzymes predominantly act on peptides. We demonstrate that one such leucine aminopeptidase is incapable of selectively removing the N-terminal methionine from methionyl-human growth hormone (Met-hGH). Some mammalian brain aminopeptidases capable of acting on low molecular weight peptides are either membrane bound or soluble enzymes, the latter having molecular weights of approximately 100,000. These enzymes often contain SH groups in addition to the essential metal atom and they are extremely unstable. All these enzymes seem to be of a rather little practical value for the "processing" of methionyl-polypeptide derivatives to mature polypeptides.

On the other hand, two microbial aminopeptidases that have been described in the literature with molecular weights of about 30,000 are promising candidates for the "processing" of met-polypeptides. The two enzymes are fairly thermostable as well as stable and optimally active at alkaline pH. The aminopeptidases most suitable for processing of Met-polypeptides are Aeromonos proteolytica aminopeptidase and Streptomyces griseus aminopeptidase.

Aeromonas aminopeptidase has been purified and characterized by Prescott and Wilkes [Methods in Enzymology 46:530-543 (1976)], and Wilkes et al., [Eur. J. Biochem, 34:459-466 (1973)]. Although they appear to demonstrate liberation of amino acids from the N-terminus of several polypeptides and

proteins, there is no demonstration of the removal of just N-terminal methionine from proteins. Removal of N-terminal methionine is demonstrated for an oligopeptide of 11 residues; however, many other residues are also removed, in addition to the methionine. Furthermore, there is no demonstration of the activity of the enzyme on non-denatured hormones of a molecular weight greater than 10,000. There is also no indication whether the reactions carried out by Wilkes and Prescott are quantitative. Also, while the paper indicates several "stop signals" for the aminopeptidase, there is no indication that the stop signals Asp or X-Pro, where X is any amino acid except for proline, are also stop signals when the enzyme is reacted with proteins. Furthermore, preliminary results indicate that not all proteins are susceptible to attack by Aeromonas proteolytica aminopeptidase. It appears that mature eucaryotic proteins are "locked" into a conformation such that the N-terminus is inaccessible to the aminopeptidase. However, the methionyl form of the eucaryotic protein has a methionine which is susceptible to removal by the aminopeptidase. Similarly eucaryotic protein derivatives containing several additional amino acids at their N-terminus, will also be susceptible to removal by the same enzyme. We have discovered that Aeromonas aminopeptidase is capable of removing the N-terminal methionine from Met-human growth hormone (Met-hGH) and from methionine - Asp-Gln-bovine growth hormone (Met-Asp-Gln-bGH). We have also demonstrated that Aeromonas aminopeptidase is capable of removing the N-terminal methionine and its adjacent leucine from Met-Leu-hGH. The reaction is quantitative and there is no other degradation of the proteins.

Summary of the Invention

A method of sequentially removing the N-terminal amino acid residues from an analog of a eucaryotic polypeptide synthesized in a foreign host comprises contacting the eucaryotic polypeptide analog with the aminopeptidase under suitable conditions permitting sequential removal of N-terminal amino acid residues. The polypeptide analog contains an amino acid residue or sequence of residues which stops the action of an aminopeptidase located at a position other than the N-terminal end of the polypeptide analog.

In preferred embodiments of the invention the foreign host in which the eucaryotic polypeptide analogs are produced is a bacterium.

The aminopeptidase enzyme used is preferably stable at a temperature up to about 65°C, and stable and active at neutral pH, i.e. about 7.0, and at an alkaline pH, i.e. from about pH 8.0 to about pH 10.0. The aminopeptidase is preferably of a molecular weight of less than about 100,000, and of bacterial origin. The enzyme can be an extracellular aminopeptidase. In specific embodiments an aminopeptidase which is insoluble in water may be used. The aminopeptidase may also be used while it is bound to a solid support, or may be removed at the end of the reaction by use of an affinity resin.

In a preferred embodiment of the invention the aminopeptidase is Aeromonas aminopeptidase. Other aminopeptidases may also be used, such as Streptomyces griseus aminopeptidase and Bacillus stearothermophilus aminopeptidase II or III.

The eucaryotic polypeptide analog may be a protein or any other peptide molecule such as apolipoprotein E, interferon, specifically gamma-interferon, or somatomedin, specifically somatomedin C.The polypeptide can also be a hormone, lymphokine, growth factor or derivatives thereof.

The N-terminal amino acid residue may be any amino acid. In specific embodiments of the invention the N-terminal amino acid residue is methionine or methionine followed by leucine. The N-terminal amino acid residue or a sequence of amino acid residues is bound to the N-terminal end of an amino acid residue or sequence of residues which acts as a stopping signal and stops the action of the aminopeptidase. In the embodiment in which Aeromonas aminopeptidase is used, the amino acid stopping signal may be an aspartic acid residue a glutamic acid residue, or a sequence of residues comprising a residue other than proline bound to the N-terminal end of a proline residue. In a specific embodiment the amino acid stopping signal comprises phenylalanine residue bound to the N-terminal end of a proline residue.

A specific embodiment of the invention involves the removal of N-terminal methionine residues from growth hormone analogs or derivatives thereof produced in bacteria. N-terminal methionine residues are removed from Met-hGH, Met-Asp-Gln-bGH, Met-bGH, and Met-pGH by contacting these growth hormone analogs with an aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

Another embodiment of the invention involves the removal of both methionine and leucine residues from the N-terminal ends of growth hormone analogs or derivatives thereof produced in bacteria. An N-terminal methionine residue and its

adjacent leucine residue are removed from Met-Leu-hGH by contacting this growth hormone analog with an aminopeptidase under suitable conditions permitting the removal of the two residues.

Another aspect of the invention is a method of adding N-terminal amino acid residues to a polypeptide molecule which comprises contacting the polypeptide molecule with an aminopeptidase and a sufficient excess of the free N-terminal amino acid to be added under suitable conditions permitting the addition of the amino acid to the N-terminus of the polypeptide. Aeromonas aminopeptidase is the preferred aminopeptidase for use in this embodiment of the invention.

In specific embodiments of the invention the Aeromonas aminopeptidase can be hyperactivated by metal substitutions of the coenzyme. In preferred embodiments Cu(II is partially substituted for Zn(II) and Ni(II) is partially substituted for Zn(II).

The invention also concerns polypeptide analogs produced by the methods of the invention. Growth hormones and analogs of growth hormones such as human and bovine growth hormones have been produced according to the methods of the invention e.g. hGH, Asp-Gln-bGH and bGH.

Another aspect of the invention is a method of preparing analogs of eucaryotic polypeptide molecules which comprises producing a first analog in bacteria by expression of a gene encoding the analog of the eucaryotic polypeptide, removing the N-terminal methionine residue and its adjacent amino acid residue by the methods of the invention with an aminopeptidase and recovering the resulting analog. The recovery of the analog can be optimized by removing the N-terminal

methionine residue and the adjacent amino acid residue that are removed by the aminopeptidase by use of ultrafiltration or dialysis.

Brief Description of the Drawings

Figure 1 shows the time course for the release of the N-terminal methionine from Met-hGH by Aeromonas proteolytica aminopeptidase as described in Example 1. By way of comparison, the release of leucine is also shown.

Figure 2 shows the time course for the release of the N-terminal methionine from Met-Asp-Gln-bGH by Aeromonas proteolytica aminopeptidase as described in Example II. By way of comparison, the release of leucine is also shown.

Detailed Description of the Invention

A method of sequentially removing one or more amino acid residues from the N-terminus of an analog of a eucaryotic polypeptide synthesized in a foreign host comprises contacting the eucaryotic polypeptide analog with an appropriate aminopeptidase under suitable conditions permitting sequential removal of N-terminal amino acid residues, the polypeptide analog containing an amino acid residue or sequence of residues located at a position other than the N-terminus of the polypeptide analog which stops the action of the aminopeptidase.

The foreign host in which the analog of the eucaryotic polypeptide is synthesized can be a bacterium or any other microorganism or organism which by use of recombinant DNA methods is capable of expressing a gene encoding for the analog and producing the resulting polypeptide.

The aminopeptidase is preferably an enzyme which remains stable at a temperature up to about 65°C. The aminopeptidase should also be stable and active at a neutral pH of about 7.0 and preferably at alkaline pH from about 8.0 to about 10.0.

In a preferred embodiment of the invention the aminopeptidase is of a molecular weight of less than about 100,000 and is of bacterial origin. The aminopeptidase can also be extracellular, insoluble in water or bound to a solid support such as agarose, or another polymeric substance. In specific embodiments of the invention an affinity resin may be used to remove excess aminopeptidase from the reaction mixture.

In the preferred embodiment of the invention the aminopeptidase is Aeromonas aminopeptidase. Other types of

aminopeptidase may also be used, e.g. Streptomyces griseus aminopeptidase, Bacillus stearothermophilus aminopeptidase II or III.

Suitable conditions permitting the removal of the N-terminal amino acid residue are known to those of ordinary skill in the art and will vary according to the type of aminopeptidase used. In the case of Aeromonas aminopeptidase suitable conditions comprise an aqueous solution at alkaline pH of about 9.5 and a temperature of about 37°C.

The eucaryotic polypeptide analog may be any polypeptide or analog of a polypeptide, such as a hormone, lymphokine, or growth factor. Suitable eucaryotic polypeptides are apolipoprotein E, interferon, namely gamma-interferon, and somatomedin, namely somatomedin C. Specific embodiments of the invention concern removing N-terminal amino acids from analogs of eucaryotic growth hormones such as human, bovine, porcine, chicken or other animal growth hormones. In these embodiments an N-terminal methionine is added to the analogs of these polypeptide growth hormones when they are produced in bacteria by recombinant DNA methods. This invention provides a method of removing the N-terminal methionine and its adjacent amino acid from human, bovine, porcine and chicken growth hormone molecules or analogs of such molecules after they have been produced in bacteria.

In certain embodiments of the invention the amino acid residue or sequence of residues which stops the action of the aminopeptidase is located adjacent the N-terminal methionine. In this situation the aminopeptidase will remove the N-terminal methionine residue only.

In another embodiment of the invention the amino acid residue or sequence of residues which stops the action of the

aminopeptidase is located adjacent the leucine in the molecule Met-Leu-hGH. In this situation, the aminopeptidase will remove both the N-terminal methionine residue and the leucine residue.

In other embodiments of the invention the residue or sequence of residues which stop the action of the aminopeptidase is separated from the N-terminal methionine by one or more amino acid residues. In this embodiment the aminopeptidase will also remove these other amino acid residues preceding the stopping signal, after it removes the N-terminal methionine.

In the case of Aeromonas aminopeptidase the amino acid residue which stops the action of the enzyme can be either aspartic acid or glutamic acid. In addition, a residue sequence comprising an amino acid other than proline bound to the N-terminus of a proline also functions as a stopping signal. In specific embodiments this stopping signal sequence comprises the amino acid phenylalanine bound to the N-terminal end of proline. This Phe-Pro sequence is the N-terminus of many natural animal growth hormone molecules. In a specific embodiment of the invention the N-terminal methionine is removed from animal growth hormone molecules produced by recombinant DNA methods in bacteria and which as a result of such production have a Met-Phe-Pro sequence at their N-terminus. In another embodiment of the invention the N-terminal methionine and its adjacent leucine residue are both removed from animal growth hormone molecules produced by recombinant DNA methods in bacteria and which as a result of such production have a Met-Leu-Phe-Pro sequence at their N terminus. In another embodiment of the invention the N-terminal methionine and its adjacent leucine residue are both removed from animal growth hormone molecules produced by recombinant DNA methods in bacteria and which as a

result of such production have a Met-Leu-Phe-Pro sequence at their N terminus.

In specific embodiments of the invention the eucaryotic polypeptides are analogs of bovine growth hormone (bGH). These analogs contain the sequences Met-Asp-Gln or Met-Phe as their N-terminal sequence. The methionine is added to the N-terminus of these growth hormones when they are produced by recombinant DNA methods in bacteria. After removal of the N-terminal methionine by aminopeptidase, Asp-Gln-bGH and bGH are recovered respectively. The bGH used in this experiment was the phenyalanine form of bGH which has a phenyalanine residue as its N-terminus in its natural state. These methods also apply, however, to removal of N-terminal methionine from the terminus of the alanine form of bGH, which contains an alanine on the N-terminus of its natural form although in this case the alanine residue may also be removed.

A preferred embodiment of the invention concerns a method of removing the N-terminal methionine residue from a eucaryotic growth hormone analog such as animal and human growth hormone analogs, produced in bacteria by expression of a gene encoding the hormone which comprises contacting the growth hormone analog with Aeromonas aminopeptidase under suitable conditions permitting removal of the N-terminal methionine residue or the N-terminal methionine residue and its adjacent leucine residue.

A specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from a human growth hormone (hGH) analog produced in bacteria by expression of a gene encoding the hormone, the human growth hormone analog having a methionine residue added to the N-terminus of authentic human growth hormone, which comprises

contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

A specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from a human growth hormone (hGH) analog produced in bacteria by expression of a gene encoding the hormone, the human growth hormone analog having a methionine residue added to the N-terminus of authentic human growth hormone, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

Another specific embodiment of the invention concerns a method of removing the N-terminal methionine residue and its adjacent leucine residue from a human growth hormone (hGH) analog produced in bacteria by expression of a gene encoding the hormone, the human growth hormone analog having a methionine residue followed by a leucine residue added to the N-terminus of authentic human growth hormone, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue and its adjacent leucine residue.

Another specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from a bovine growth hormone analog produced in bacteria by expression of a gene encoding the bovine growth hormone analog, the bovine growth hormone analog having a methionine residue added to its N-terminus, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

Another specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from an interferon analog, such as gamma-interferon, produced in bacteria by expression of a gene encoding the interferon analog which comprises contacting the interferon analog with Aeromonas aminopeptidase under suitable conditions permitting removal of the N-terminal methionine residue.

Another specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from a somatomedin analog, such as somatomedin C, produced in bacteria by expression of a gene encoding the somatomedin analog, the somatomedin analog having a methionine residue added to the N-terminus, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

Another specific embodiment of the invention concerns a method of removing the N-terminal methionine residue from an apolipoprotein E analog produced in bacteria by expression of a gene encoding the analog, the analog having a methionine residue added to the N-terminus, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

The invention also concerns a method of adding an N-terminal amino acid residue to a polypeptide molecule which comprises contacting the polypeptide molecule with an aminopeptidase and a sufficient excess of the free N-terminal amino acid residue to be added under suitable conditions permitting addition of the amino acid to the N-terminus of the polypeptide. Any aminopeptidase enzyme may be used; however,

Aeromonas aminopeptidase is preferred. The aminopeptidase used will be able to add any amino acid residue to the N-terminus of the polypeptide as long as that amino acid does not function as a stopping signal for the enzyme and it would preferably add an amino acid to the N-terminus which serves as a stopping signal. Since the aminopeptidase reaction is a reversible reaction, the conditions for the addition reaction are the same as that of the cleavage reaction except for the concentration of the free amino acid to be added.

The activity of the Aeromonas aminopeptidase can be increased by metal substitutions. The greatest enhancement of activity occurs by partial or mixed metal substitutions essentially according to the methods J.M. Prescott et. al., Biochemical and Biophysical Research Communications, Vol. 114, No. (pp. 646-652) 2 (1983). The partial or mixed metal substitutions may be Cu(II) for Zn(II) or Ni(II) for Zn(II).

The invention also concerns polypeptide analogs produced by the methods of this invention such as human, bovine, porcine and chicken growth hormones or growth hormone analogs such as Asp-Gln-bGH.

Another aspect of the invention is a method of preparing an analog of a eucaryotic polypeptide which comprises providing a first analog in bacteria by expression of a gene encoding the analog of the eucaryotic polypeptide. The N-terminal methionine residue or an N-terminal methionine residue and its adjacent leucine residue of this analog is then removed by the method of this invention with an aminopeptidase, e.g. Aeromonas aminopeptidase. The resulting analog is then recovered by using methods known to those of ordinary skill in the art.

The recovery of the analog may be optimized by removing the free N-terminal methionine and leucine residues cleaved from the polypeptide analog by the aminopeptidase. Removal of the free amino acids drives the reaction to completion. The removal may be by any method known to those of ordinary skill in the art, e.g. ultrafiltration or dialysis. The invention also concerns analogs of eucaryotic polypeptides prepared by the methods of this invention such as growth hormones, e.g. human, bovine, and porcine growth hormone.

EXPERIMENTAL DETAILS

Materials and Methods

Met-hGH and Met-Asp-Gln-bGH were prepared by recombinant DNA techniques. Coomasie blue staining of polyacrylamide gels (15% gels) of the proteins, electrophoresed in the presence of sodium dodecyl sulfate and 2-mercaptoethanol reveals: a) a major band of Mw approximately 22,000 corresponding to Met-Asp-Gln-bGH and a very faint band with a slightly higher (lot 108-) (Bio-Technology General (Israel) Ltd.) or lower (lot 113D) (Bio-Technology General (Israel) Ltd.) molecular weight for the Met-Asp-Gln-bGH molecules; b) major band Mw approximately 22,000 and a very faint band with a slightly lower molecular weight for the Met-hGH (lot 1/100) molecule (Coomassie Blue). Scanning of S.D.S. gels reveals 88-93% purity for both proteins. Purity of 95% and greater has been obtained in other preparations. No detectable contaminating endopeptidase activity was present as judged by electrophoresis of the proteins on SDS-polyacrylamide gels after 24 hour incubation at 37°C.

Aeromonas aminopeptidase was prepared from the extracellular filtrate of Aeromonas proteolytica obtained from the American Type Culture Collection (ATCC 15338), essentially according to Prescott, J.M. and Wilkes, S.H., Methods Enzymol. 46: 530-543 (1976). The purification procedure included the following steps: sedimentation and filtration of bacteria, ammonium sulfate precipitation of the filtrate (367g per liter), acetone fractionation (43.7% to 70% acetone), heat treatment at 70°C (for 8 hrs.) to destroy endopeptidase activity, gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Sephadex A-50. In all experiments 10 mM Tris-HCl buffer, pH 8.0 replaced 10 mM tricine buffer, pH 8.0 employed by Prescott and Wilkes.

Preequilibration and elution from the G-75 column were performed in the presence of 5 micromoles $ZnCl_2$ rather than 50 micromoles $ZnCl_2$ employed in the original procedure. Purification on the DEAE-Sephadex A-50 column was performed by preequilibration of the column with 0.1M NaCl in 10 mM Tris-HCl, pH 8.0 containing 5 micromoles $ZnCl_2$, application of the sample and gradient elution with 0.6 M NaCl in the same buffer (containing 5 micromoles $ZnCl_2$). After the salt concentration increased to about 0.5M the column was eluted with 0.7M NaCl in the same buffer. The major peak which eluted from the column was collected and dialyzed against 10 mM Tris-HCl, 0.1 M NaCl, pH 8.0 containing 5 micromoles $ZnCl_2$ and then kept frozen to -20°C.

Prior to reaction with the growth hormones the enzyme solution was incubated at 70° for 2 hrs. to inactivate any possible traces of endopeptidase activity that might have been retained in the preparation and reactivated after prolonged storage. For large-scale experiments the enzyme was incubated at 70°C for 3 h prior to reaction with the hormone.

Amino acid analysis was performed on a Dionex D-502 amino acid analyzer. Amino acid sequence analysis was carried out with an Applied Biosystems Gas Phase Sequencer followed by high performance liquid chromatography of the PTH-amino acids.

Example I

Time dependence of the release of free methionine from Met-hGH by Aeromonas aminopeptidase

Prior to reaction with Met-hGH a sample of the aminopeptidase eluted from the DEAE-Sephadex A-50 column, 0.63 mg/ml in 10 mM Tris-HCl, 0.1M NaCl, pH 8.0, was incubated at 70°C for 2h to inactivate traces of endopeptidase activity. The enzyme was then diluted 3:1 with 2M Tris-HCl, pH 9.5 to a final concentration of 0.4725 mg/ml enzyme.

Met-hGH was dissolved to 8mg/ml (by weight) in 10mM Na Borate, pH 9.5.

Nine hundred microliters of Met-hGH solution and 19 microliters of the aminopeptidase solution were mixed and incubated at 37°. 50 microliter aliquots were taken after 2 min, 5 min, 10 min, 15 min, 30 min, 60 min, 2h, 4h and 22h and precipitated by adding an equal volume of 3% sulfosalicylic acid solution in water, incubating at 37° for 15 min and then centrifuging in an Eppendorf bench centrifuge. 50 microliter samples of the supernatant were taken for direct amino acid analysis (without acid hydrolysis). Control experiments were run by precipitating Met-hGH solution (8 mg/ml) alone, directly after dissolution at time t_o or after incubation of the Met-hGH alone at 37° for 4h and for 22h. Again for each of the controls 50 microliters of hormone solution was precipitated with an equal volume of 3% sulfosalicylic acid and 50 microliters of the supernatant were taken for amino acid analysis. Assuming a molecular weight of approximately 21,800 and that 85% of the weighed material is hormone (5%-10% water, 90%-95% purity of hormone), each analysis corresponds to 7.63 nmoles of Met-hGH starting material. The amount of methionine and several other amino

acids liberated by the enzyme are listed in Table 1.

The release of methionine and that of leucine are depicted as a function of time in Figure 1. The N-terminal sequence of Met-hGH is shown in Table IV. Polyacrylamide gel electrophoresis of the products reveals no detectable degradation of the hGH.

TABLE I

Analysis of Methionine and Some Other Amino Acids
Released from the Digest of
Met-hGH by Aeromonas Aminopeptidase
(nmoles 50 microliter sample)

	1	2	3	4	5	6	7	8	9	10	11	12
										Met-hGH	Met-HGH	Met-hGH
	2 min	5 min	10 min	15 min	30 min	60 min	2h	4h	22h	t_o	4h	22h
Met	3.95	6.55	7.88	8.05	8.35	8.05	8.13	8.65	7.76	less than 0.20		
Asp	0.23	0.15	0.16	0.13	0.11	0.15	0.21	0.22	0.31	less than 0.20		
Gly	0.28	0.25	0.31	0.21	4.48*	0.26	0.37	0.34	0.35	0.16	0.26	0.40
Ala	0.20	0.18	0.21	0.16	0.13	0.20	0.28	0.27	0.20	0.10	0.11	0.22
Ile	0.07	0.05	0.06	0.05	0.05	0.07	0.09	0.10	0.14	less than 0.20		
Leu	0.18	0.13	0.14	0.13	0.13	0.17	0.22	0.25	0.35	less than 0.20		
Phe	less than 0.2.....											

*Most probably a contamination of the analysis sample

Neutral protease contaminants of aminopeptidase prefer to cleave internal peptide bonds on the amino side of hydrophobic amino acids. This reveals, for example, Leu and Ile residues which are then liberated by the aminopeptidases. The release of other amino acids not listed are also negligible.

Example II

Time dependence of the release of free methionine from Met-Asp-Gln-bGH by Aeromonas aminopeptidase

Prior to reaction with Met-Asp-Gln-bGH a sample of the aminopeptidase eluted from the DEAE-Sephadex A-50 column was heated at 70°C and diluted as described in Example I.

Met-Asp-Gln-bGH was dissolved to 8mg/ml (by weight) in 10 mM Na Borate, pH 9.5.

750 microliters of Met-Asp-Gln-bGH solution and 32 microliters of the aminopeptidase solution were mixed and incubated at 37°. 50 microliter aliquots were taken after 5 min, 10 min, 15 min, 30 min, 60 min, 2h, 4h and 22h and precipitated by adding an equal volume of 3% sulfosalicylic acid solution in water, incubating at 37°C for 15 min and centrifuging in an Eppendorf bench centrifuge. Again 50 microliter samples were taken for amino acid analysis. Control experiments were run by precipitating Met-Asp-Gln-bGH solution (8 mg/ml) alone either directly after dissolution at t_o or after incubation at 37°C for 22h. Precipitation of protein and amino acid analysis of the supernatant were carried out as described in Example 1. Assuming a molecular weight of approximately 22,000 and that 85% of the weighted material is hormone (5-10% water, 90-95% purity of hormone), each analysis corresponds to 7.41 nmoles Met-Asp-Gln-bGH starting material. The amount of methionine and several other amino acids liberated are listed in Table II.

The release of free methionine as well as of leucine as a function of time is depicted in Figure 2. The N-terminus sequence Met-Asp-Gln-bGH is shown in Table IV.

Polyacrylamide gel electroporesis of the products reveals no detectable degradation of bGH analog.

Table II

Analysis of Methionine and Some Other Amino Acids
Released from the Digest of
Met-Asp-Gln-bGH by Aeromonas Aminopeptidase

(nmoles 50 ul sample)

	1	2	3	4	5	6	7	8	9	10
									Met-Asp-Gln-bGH	
	5 min	10 min	15 min	30 min	60 min	2h	4h	22h	t_o	22h
Met	2.87	4.06	5.04	6.28	6.63	6.81	7.33	7.69	less than 0.20	
Asp	0.13	0.13	0.11	0.14	0.14	0.15	0.19	0.28	less than 0.20	
Gly	0.16	0.19	0.15	0.27	0.19	0.19	0.19	0.26	less than 0.20	
Ala	0.17	0.19	0.18	0.22	0.23	0.25	0.28	0.44	less than 0.20	
Ile	0.05	0.06	0.06	0.07	0.07	0.09	0.10	0.21	less than 0.20	
Leu	0.10	0.11	0.12	0.15	0.18	0.22	0.27	0.56	less than 0.20	
Phe	less than 0.20.....						0.12	0.28	less than 0.20	

Neutral protease contaminants of aminopeptidases prefer to cleave internal peptide bonds on the amino side of hydrophobic amino acids. This reveals, for example, Leu and Ile residues which are then liberated by the aminopeptidases. The release of other amino acids not listed are also negligible.

Example III

Comparison of Aeromonas aminopeptidase and leucine aminopeptidase (Microsomal from porcine kidney, Sigma L5006)

Aeromonas aminopeptidase, 0.63 mg/ml in 10 mM Tris-HCl, 0.1 M NaCl, pH 8.0 was incubated at 70°C for 2h to inactivate traces of endopeptidase activity. The enzyme was then diluted 3:1 with 2M Tris-HCl, pH 9.5 to a final concentration of 0.4725 mg/ml.

Leucine aminopeptidase (porcine kidney, microsomal, Sigma L5006), 1 mg/ml suspension in 3.5 M $(NH_4)_2 SO_4$, 10 mM $MgCl_2$, pH 7.7, 100 microliters was mixed with 0.5M Tris HCl, pH 9.5, 25 microliters H_2O, 150 microliters, and 0.025 M $MnCl_2$, 25 microliters, and the mixture incubated at 37°C for 2h.

Met-hGH, was dissolved 11 mg/ml in 10 mM NaBorate, pH 9.5.

1) 290 microliters of Met-hGH solution, 11 mg/ml + 110 microliters 10 mM Na Borate, pH 9.5 + 17 microliters Aeromonas aminopeptidase solution (final enzyme concentration: 19.3 micrograms/ml),

or

2) 400 microliters of Met-hGH solution, 11 mg/ml + 85 microliters leucine aminopeptidase activated enzyme + 85 microliters 0.125 M $MgCl_2$ (final incubated enzyme concentration: 49.7 micrograms/ml),

were incubated at 37°C and 75 microliter aliquots were taken

after 5 min, 3h and 22 h and precipitated with an equal volume of 3% sulfosalicylic acid. After incubation at 37°C for 15 min the mixture was centrifuged and 50 microliters of the supernatant were taken for direct amino acid analysis. Assuming a molecular weight of approximately 21,800 and that 85% of the weighed material is the hormone, each analysis corresponds to 7.46 nmoles and 7.53 nmoles Met-hGH of starting material for the reaction with the Aeromonas enzyme and the procine leucine aminopeptides, respectively.

Control experiments were run by precipitating Met-hGH with an equal volume of 3% sulfosalicylic acid after dissolution or after 22h of incubation at 37°C. The precipitated mixture was incubated for 15 min at 37°C, centrifuged, and 50 microliters of the supernatant taken for direct amino acid analysis. The results of the experiment are given in Table III. These results show that the leucine aminopeptidase does not remove the N-terminal methionine and that the small amount of methionine released is likely due to the release from small peptides formed by contaminants of endopeptidase activity (see amounts of Ile and Leu). This conclusion is confirmed by polyacrylamide gel electrophoresis showing some degradation of the hormone by the enzyme after 22h incubation at 37°C.

TABLE III

Release of N-Terminal Methionine from Met-hGH by Aeromonas Aminopeptidase and by Porcine Kidney Leucine Aminopeptidase (Microsomal) (nmoles/50 microliter sample)

			Met	Ile	Leu
Met-hGH at t_0 (1)			less	than	0.1
Met-hGH 22h at 37° C (2)			less	than	0.1
Met-hGH + Aeromonas Aminopeptidase	5 min	(1)	6.76	less than	0.1
Met-hGH + Aeromonas Aminopeptidase	3h	(2)	7.11	0.09	0.19
Met-hGH + Aeromonas Aminopeptidase	22h	(3)	7.27	0.10	0.28
Met-hGh + Leucine Aminopeptidase	5 min	(1)	less	than	0.1
Met-hGH + Leucine Aminopeptidase	3h	(2)	0.25	0.09	0.27
Met-hGH + Leucine Aminopeptidase	22h	(3)	0.62	0.78	1.12

Neutral protease contaminants of aminopeptidases prefer to cleave internal peptide bonds on the amino side of hydrophobic amino acids. This reveals for example Leu and Ile residues which are then liberated by the aminopeptidases.

Example IV

Removal of N-terminal methionine from Met-Asp-Gln-bGh and preparation of the sample for sequence analysis.

2.5 ml Met-Asp-Gln-bGH, 8mg/ml in 10 mN NaBorate, pH 9.5, was incubated with 106 microliters enzyme, 0.4725 mg/ml in 0.5 M Tris-HCl, pH 9.5, 22h at 37°C. For the determination of the amino terminal sequence, 2 ml of the mixture were diluted 1:1 with 10 mM Na Borate, pH 9.5 and 1 ml 15% sulfosalicylic acid were added, the mixture incubated at 37°C for 15 min and precipitated by centrifugation. The pellet was resuspended in 5 ml 3% sulfosalicylic acid and recentrifuged. The pellet was suspended in 5 ml 10 mM Na Borate, pH 10.5 and dialyzed against three 2 liter changes of water containing 1 ml, 0.3 ml and 1 ml of concentrated ammonium hydroxide, respectively, and then was dialyzed against water. The sample was brought to 20% acetic acid (by glacial acetic acid) and used for sequence analysis. The results of the sequence analysis, shown in Table IV, demonstrate that more than 95% of the molecules have the N-terminal sequence Asp-Gln-Phe-Pro.

Table IV

N-Terminal Sequences of Growth Hormone Derivatives Before and After Removal of N-Terminal Methionine by Aeromonas Aminopeptidase

Growth Hormone Derivative	N-terminal Sequence[4]	N-terminus(%)[5]
1) Met-Asp-Gln-bGH[1]	Met-Asp-Gln-Phe-Pro	Met (90-100)[6]
2) Asp-Gln-bGH[2]	Asp-Gln-Phe-Pro	Asp (95)[7]
3) Met-hGH[1]	Met-Phe-Pro	Met (90-100)[6]
4) hGH[3]	Phe-Pro	Phe (99)[7]

[1] Bovine growth hormone analog. [2] Obtained from derivative (1) in example IV. [3] Obtained from derivative (3) as described in example V. [4] Amino acid sequence analysis was carried out with an Applied Biosystems Gas Phase Sequencer followed by high performance liquid chromotography of the PTH-amino acids. [5] Determined by sequence analysis. [6] The amount of methionine at the N-terminus of the starting products was estimated by the amount of free methionine liberated by reaction with the Aeromonas aminopeptidase after precipitation with sulfosalycilic acid and analysing the supernatant by amino acid analysis. [7] The amino acid present at the N-terminus of the products was estimated from PTH-amino acid analysis of the 1st cycle of the sequence analysis.

Example V

Use of ultrafiltration or dialysis to drive the reactions

The reactions carried out by the enzyme are reversible. Thus, removal of one of the products will tend to further drive the reaction to completion. We have demonstrated this by removing the liberated methionine residue during the course of the reaction, thus driving the reaction to further produce hGH. The liberated methionine residue was eliminated by ultrafiltration.

Twelve grams of Met-hGH in 1500 ml, 10 mM NaBorate, pH 9.5, was incubated with 12.4 ml of enzyme 0.4725 mg/ml in 0.5 M Tris-HCl, pH 9.5, at 37°C for 2h. An additional amount of 6.2 ml of the same enzyme solution was added and incubation at 37°C was continued for 3 1/2 h. The solution was placed for ultrafiltration and about 50 liters of 10 mM NaBorate, pH 9.5, were passed through the material during 4h to remove free methionine and drive the enzymatic reaction to completion. Incubation at 37°C was then continued for 12 1/2 h. Total duration of the incubation and ultrafiltration was 22h. The material was absorbed on DEAE-Sephacel and the resin washed with 10 mM NaBorate, pH 9.0 and then with 10 mM NaBorate, pH 9.0 containing 25 mM NaCl, 50 mM NaCl and 75 mM NaCl. The hormone was eluted with 10 mM NaBorate, pH 9.0, containing 100 mM NaCl. The eluted hormone was concentrated, and dialyzed by ultrafiltration and lyophilized. A sample was dissolved in 20% acetic acid and subjected to sequence analysis. The results of analysis are shown in Table IV. The results demonstrate that in more than 99% of the molecules, the N-terminal methionine was removed and there was no further degradation of the protein.

Example VI

Time dependence of the release of free methionine and free leucine from Met-Leu-hGH by Aeromonas aminopeptidase

Prior to reaction with Met-Leu-hGH, a sample of the aminopeptidase eluted from the DEAE-Sephadex A-50 column was heated at 70°C and diluted as described in Example I.

Met-Leu-hGH was dissolved to 8 mg/ml (by weight) in 10 mM Na Borate buffer, pH 9.0. The pH was raised to 10.6, then dropped to 8.8, and finally the mixture was centrifuged to remove a small amount of precipitate. The supernatant solution was used for reaction.

One thousand microliters of the Met-Leu-hGH solution and 21 microliters of the Aeromonas aminopeptidase were mixed and incubated at 37°. Seventy-five microliter aliquots were taken after 2 min., 5 min., 10 min., 30 min., 60 min., 2 h. and 22 h. and precipitated by adding an equal volume of 3% sulfosalicylic acid solution in water, incubating at 37° for 15 min. and then centrifuging in an Eppendorf bench centrifuge. Fifty microliter samples of the supernatant were taken for direct amino acid analysis (without acid hydrolysis). Control experiments were run by precipitating the Met-Leu-hGH solution (8 mg/ml) alone with an equal volume of 3% sulfosalicylic acid, at a time t_o or after incubating the Met-Leu-hGH solution at 37° for 22 h. Again 50 microliters of the supernatant solutions were taken for direct amino acid analysis. The results of this experiment are summarized in Table V.

The release of methionine and that of leucine are depicted as a function of time in Figure 3. The N-terminal sequence of Met-hGH is shown in Table IV. Polyacrylamide gel elec-

trophoresis of the products reveals no detectable degradation of the hGH.

Table V

Analysis of Methionine and Leucine As Well as Some Other Amino Acids Released from the Digest of Met-Leu-hGH by Aeromonas Aminopeptidase

(nmoles 50 microliter sample)

	1	2	3	4	5	6	7	8	9	10
	2 min	5 min	10 min	15 min*	30 min	60 min	2 h	22 h	t_o	22 h
Met	5.15	4.85	4.83	2.94	5.28	5.30	4.85	4.70	less than 0.2	0.32
Leu	4.80	4.58	4.47	2.73	5.10	5.22	4.71	5.52	less than 0.2	0.10
Asp	less than 0.2								0.2	less than 0.2
Gly	less than 0.2									
Ala	less than 0.2								0.23	0.2
Ile	less than 0.2									
Phe	less than 0.2									

*Most probably a pipetting error.

The release of other amino acids not listed is also negligible.

EXAMPLE VII

Removal of Met from a commercial preparation of Met gamma-Interferon.

Thirty microliters of gamma-interferon (Amgen; Interferon-gamma-4A; ARN 3010, batch 1), containing the authentic sequence of the lymphokine and having a specific activity of 1-5 x 10^7 units/mg, was subjected to microsequence analysis. Analysis of the first three amino acids indicated that the material contains Met-gamma-Interferon with the N-terminal sequence Met-Gln-Asp (with some trace of Arg found in the third cycle).

This gama-interferon derivative was acted upon by the Aeromonas aminopeptidase and found to release free methionine as determined by amino acid analysis using a particularly sensitive amino acid analyzer with picomole sensitivity and ortho-phthalaldehyde post-column derivatization. Note that all other amino acid analyses in the application were carried out at nanomole sensitivities using a Dionex D-502 amino acid analyzer. All sequence analyses were carried out on an Applied Biosystems Model 470A protein sequencer and followed by HPLC of the PTH-amino acids. The procedure of the removal of the methionine is as follows:

Methionyl-gamma-interferon: Interferon-gamma$_{4A}$ ARN 3010, batch 1, 10^7 units/ml (1-5 x 10^7 units/mg) in 0.04 M Tris·HCl, ph 7.0.

Aeromonas aminopeptidase: (Lot 2), 0.5 mg/ml in 0.1 M NaCl-10 mM Tris-HCl 5 micromoles $ZnCl_2$, ph 8.0 was heated at 70° for 2 h, prior to use. It was then diluted 1:9 with 0.1 m

NaCl, 10 mM Tris·HCL, 5 micromoles $ZnSO_4$, pH 8.0, to 0.05 mg/ml enzyme.

Procedure: 12 microliters of the gamma-interferon solution and 3 microliters of the enzyme solution (0.05 mg/l) were incubated at 37°C for 35 min and the mixture cooled on ice. After 30 min, 14 microliters of the mixture were dried by lyophilization and loaded on the amino acid analysis column without further treatment. Control experiments were run by incubating 12 microliters of the gamma-interferon alone and 3 microliters of the enzyme alone, at 37°C for 35 min, drying the samples as above and applying them on the amino acid analysis column.

The amount of methionine released was 96 picomoles and the background of other amino acids was fairly normal: Asp, 15 picomoles; Thr, 24 picomoles; Ser, 39 picomoles; Glu, 8 picomoles; Gly, 53 picomoles; Ala, 23 picomoles; Val, 10 picomoles; Leu, 8 picomoles and Phe, 11 picomoles; there was another large contaminating peak, at the position where the enzyme reference also showed a peak. Relatively large background peaks of Ser and Gly were also seen on the gamma-interferon reference. Assuming that the specific activity of the sample is $5x10^7$ units/mg and mol wt. for gamma-interferon of approximately 17,000, the amount of methionine released amounts to 73% of the theoretical value. If the specific activity of the material is lower than the above assured value, percentage of removal of Met could be lower.

This experiment indicates that the N-terminal methionine can be selectively and efficiently removed from Met-gamma-interferon by Aeromonas aminopeptidase.

EXAMPLE VIII

Removal of an N-terminal Met from an interferon

A recombinant interferon analog having a Met at its N-terminus was processed according to the method of the present invention. Met was selectively removed from the N-terminus of the molecule by the Aeromonas aminopeptidase.

EXAMPLE IX

Removal of an N-terminal Met from a Somatomedin C polypeptide

A recombinant somatomedin C polypeptide having a Met at its N-terminus was processed according to the method of the present invention. Met was selectively removed from the N-terminus of the molecule by the Aeromonas aminopeptidase.

EXAMPLE X

Removal of Met from Met-Porcine Growth Hormone (PGH) and non-removal of N-terminal Ala from the mature recombinant Cu_2-Zn_2 human superoxide dismutase.

Aeromonas aminopeptidase lot 2, 0.5 mg/ml (in 0.1M NaCl-10mM Tris HCl pH 8.0 was heated at 70°C for 2 hours prior to use, then diluted 3:1 with 2M Tris HCl, pH 9.5.

Met-PGH (lot 5/100) and Cu_2-Zn_2 human superoxide dismutase (SOD lot 1) were prepared by recombinant techniques. The latter has the authentic N-terminal sequence of the mature protein except that the N-terminal Ala is not N-Acetylated.

Procedure: The proteins were dissolved, 8 mg/ml in 10mM sodium borate, pH 9.5. To 600 microliters of the protein solutions were added 34 microliters of the enzyme solution and the mixtures were incubated at 37°C. Samples were taken with time and precipitated with equal volume of 3% sulfosalicylic acid and incubated at 37°C for 15 minutes, then centrifuged. 50 microliters of the supernatant solutions were taken for amino acid analysis. Control experiments were run by incubating the proteins alone at 37°C for 22h and proceeding with the amino acid analyses as above. Assuming 85% content of the weighed proteins and molecular weights of 22,000 and 16,000 (per subunit) for Met-PGH and Cu_2-Zn_2 superoxide dismutase, respectively, the theoretical amount of N-terminal residues in each analysis are 7.31 nmoles and 10.17 nmoles, respectively. The amount of Met and Ala released are shown in Table VI.

TABLE VI

Release of Met for Met-PGH and Ala for recombinant Cu_2-Zn_2 human superoxide dismutase by Aeromonas aminopeptidase*

Met-PGH	30 min	3 hr	22 hr	t22 hr (control)
Met released	1.95	2.41	4.63	0.11

Recombinant Cu_2-Zn_2 human superoxide dismutase	30 min	3 hr	22 hr	t22 hr (control)
Ala released	.15	.32	.53	0.10

*Under the same conditions, the release of Met from Met-hGH was essentially quantitative in 3 hours.

There could be several reasons for the non-stoichiometric removal of Met from Met-PGH (in contrast to the stoichiometric removal of Met from Met-hGH). One explanation could be that the molecules could be present as non-covalently associated dimers and that the N-terminal methionine of only one of the molecules in the dimer is accessible to the enzyme attack, whereas the N-terminal methionine of the other molecule in the dimer is sterically hindered. For this reason, only about 50% - 60% of the N-terminal methionine residues were removed. Met-hGh, on the other hand, is monomeric, Another possibility is that in Met-pGH part of the molecules are still formylated and the enzyme does not remove formyl-methionine. In order to prove any of these or other possibilities further experiments would be required.

EXAMPLE XI

Removal of Met, Lys and Val from Apolipoprotein E by Aeromonas Aminopeptidase

Methionyl-Apolipoprotein E (Lot CC 017) with the N-terminal sequence Met-Lys-Val-Glu was prepared in E. coli and purified. It was used as a solution of 2.53 mg/ml in 5mM NH_4HCO_3.

Aminopeptidase. Aeromonas aminopeptidase (Lot 2) was used in the experiment. The enzyme, 0.5 mg/ml in 0.1 M NaCl-10 mM Tris-HCl-5 micromoles $ZnCl_2$, pH 8.0 was heated for 2.5 hours prior to reaction with the protein.

Procedure. 600 microliters of Methionyl-Apolipoprotein E and 12.25 microliters of enzyme were incubated at 37°C and 90 microliter aliquots of the mixture were taken with time and precipated with 10 microliters of 15% sulfosalycilic acid in water. The mixture was incubated at 37°C for 15 minutes and centrifuged. 50 microliters of the supernatant were taken for direct amino acid analysis (without acid hydrolysis). A control experiment was run by incubating the protein alone without the enzyme for 22 hours, and proceeding with the analysis as above. The amounts of methionine, lysine and valine released from the protein are given in Table VII.

TABLE VII

Release of Met, Lys and Val from Methionyl-Apolipoprotein E by Aeromonas Aminopeptidase

	(nmoles amino acid released)					
	10 min	30 min	1 h	4 h	22 h	t_{22} (control)
Met	2.64	2.77	2.73	2.88	3.09	less than 0.1
Lys	2.11	2.37	2.44	2.64	2.80	"
Val	1.87	2.19	2.10	2.41	3.22	"

The amount of methionine, lysine and valine released agree with the theoretical amount expected, based on the specified concentration of the sample and assuming a molecular weight of approximately 35,000 for the protein (i.e. 3.19 nmoles each). Yet, the removal of the third amino acid, Val is somewhat slower than the other amino acids. Within the first 1 hour of reaction no release of Glutamic acid could be observed indicating the stopping character of this amino acid for the aminopeptidase. After 22 hours of incubation, a small amount of degradation of the protein could be observed on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. This could reflect traces of endopeptidase activity in either the substrate or the enzyme. The SDS gels show that the new ApoE derivative, without the three amino acids Met, Lys, and Val, migrates slightly faster than the parent protein. Interestingly, the enzyme in the reaction mixture lost activity after incubation for 22 hours, probably caused by the Apolipoprotein E which is cytotoxic and may also inactivate the enzyme. With all other substrates tested

so far, the enzyme activity in the reaction mixture is fairly well preserved after 22 hours at 37°.

EXAMPLE XII

Removal of Met from Met-bGH

This example shows the removal of N-terminal methionine from Methionyl-bGH, where bGH is the phenylalanine form of bGH, having the N-terminal sequence Met-Phe-Pro.

Met-bGH (Lot 178) was prepared in E. coli for the purpose of the present experiments.

Aminopeptidase. Aeromonas aminopeptidase (Lot 2), 0.5 mg/ml in 0.1 M NaCl - 10 mM Tris - HCl, 5 micromole $ZnCl_2$, pH 8.0 was preheated at 70°C for 2 h prior to use, then diluted 3:1 with 2 M Tris HCl, pH 9.5 to a final concentration of 0.375 mg/ml.

Procedure The hormone was suspended, 8 mg/ml, in 10 mM sodium borate buffer, pH 9.5, the pH was raised to 12 with 1N NaOH, then lowered back to pH 9.4 with 1N HCl, then centrifuged to remove a slight amount of precipitate, and yielded a solution of approximately 7 mg/ml.

One thousand microliters of the Met-bGH solution and 56.3 microliters of the enzyme solution were incubated at 37°C and 75 microliter aliquots of the mixture were taken with time and precipitated with an equal volume of 3% sulfosalycilic acid. After incubation of the mixture at 37°C for 15 min. the precipitate was centrifuged and 50 microliters of the supernatant taken for direct amino acid analysis (without acid hydrolysis). Control experiments were run by precipitating the hormone alone at time zero or after incubation at 37°C for 22 h and proceeding with the analyses as above. The results of the experiment are given in Table VIII.

TABLE VIII

Release of Met from Methionyl-bGH by Aeromonas Aminopeptidase

(nmoles Met/50 ul sample)

								Control	
2 min	5 min	10 min	30 min	1 h	2 h	4 h	22 h	t_0	t_{22}
3.48	3.67	3.76	3.77	3.77	3.81	4.19	4.45	0.1	0.29

The experiment demonstrates that the reaction of Aeromonas aminopeptidase with Met-bGH is very rapid since at 20 micrograms/ml of enzyme most of the reaction is complete in 2 min.

The stoichiometry of the reaction is about 65% as opposed to stoichiometry of 90-100% which were observed for the reaction of the enzyme in several reactions with two different batches of Met-hGh, with Met-Asp-Gln-bGH and with Met-Apolipoprotein E as well as with Met-gamma-interferon and Met-somatomedin. On the other hand, reaction with Met-Leu-hGH and Met-bGH showed only approximately 65% of Met released/mole substrate and with Met-pGH only approximately 50%-60%. We have recently observed that with certain new batches of Met-Asp-Gln-bGH the stoichiometry is also in the 50-60% range. We previously assumed that this partial stoichiometry would be due to either a) not completely pure materials; b) incomplete removal of the N-formyl group by the E. coli host deformylating enzyme(s) and/or; c) to dimer formation of some hormones and accessibility of the enzyme to only one of the monomers in the dimer. We found indications for yet another likely explanation for the incomplete stoichiometry, namely, that the host E. coli processing enzyme system partially removes

some of the N-terminal methionine, e.g. in Met-pGH and Met-bGH, before purification of the proteins.

EXAMPLE XIII

Biological Activity of Authentic Recombinant hGH, Obtained from Met-hGH by Removal of Met with Aeromonas Aminopeptides

The authentic recombinant hGH obtained from Met-hGH by reaction with Aeromonas aminopeptidase by procedures essentially the same as those described in Examples 1 and 2 including use of ultrafiltration to remove free methionine is biologically active and displays high activity. Thus, the batch preparation of hGH described in Example 1 and 2 (lot 2/100), that was derived from Met-hGH lot 1/100 has an N-terminal Phe. Its immunoreactivity is the same as that of pituitary hormone from frozed glands and its biological activity by radioreceptor binding assay is 2.1 IU/mg. In addition, another batch preparation of Met-hGH (lot 4.1.1) that was passed on an anion-exchange column to remove deamidated froms of the hormone, then treated with the Aeromonas aminopeptidase, using the ultrafiltration techniques to remove free methionine that was released during the reaction in order to drive the reaction to completion, then passed on another column of an anion exchanger and lyophilized, was designated lot 4.2.1 and analyzed. The results were:

a) The first 38 amino acids for N-terminus wre identical to that of the natural pituitary derived product, with the amino acid at the N-terminus being Phe (at least 99%).

b) The C-terminal residue was Phe, also identical to that of the pituitary derived product.

c) The immunoreactivity is 1.35 times higher than that of a commercial preparation from pituitary.

d) The activity by radio-receptor binding assay is 2.5 units/mg protein.

Discussion

The experiments and results presented above clearly demonstrate that Aeromonas aminopeptidase rapidly removes the N-terminal methionyl residue from Met-hGH, Met-Asp- Gln-bGH, Met-gamma-interferon, Met-Somatomedin C, Met-pGH, Met-Apolipoprotein E, and Met-bGH molecules prepared by recombinant DNA techniques. Also, the aminopeptidase can remove the N-terminal methionine residue and its adjacent leucine residue from Met-Leu-hGH, prepared by recombinant DNA techniques. Precautions taken to avoid endopeptidase activity both from the substrate and the aminopeptidase have proven successful in the sense that the enzymatic reactions contain very little if any detectable endopeptidase cleavages (Figures. 1 and 2 and Tables I-VIII) even after 22h of incubation of the hormones with the aminopeptidase. Thus, conditions under which completion of the enzymatic reaction takes place without significant endopeptidase activity are readily available.

Additionally, the results of Example VI demonstrate that Aeromonas aminopeptidase can rapidly remove several amino acids from eucaryotic polypeptide analogs, not only one methionyl residue. In particular, it has been demonstrated that the N-terminal methionyl and leucyl residues can be removed from Met-Leu-hGH to yield authentic human growth hormone.

The most striking conclusion of the experiment of Example VI is that the amount of methionine and leucine released is the same, even after only two minutes of reaction. This is due to the fact that the leucine residue is probably being removed at a faster rate than the methionine residue. Thus, a recombinant DNA product of the design Met-Leu-hGH, where Met is followed by Leu, assures that the final product will be hGH with no detectable presence of Leu-hGH molecules.

This experiment demonstrates that the authentic molecule can be obtained not only from a methionyl-derivative but also from a methionyl-x-derivative where x is another amino acid. Similarly, an authentic molecule would be obtained from $(x)_n$-derivative where n is greater than two.

The enzymatic reaction is specific. In the two reactions examined, there are clear Asp and X-Pro stops of the aminopeptidase that are in accord with the specificity of the enzyme towards small peptides.

The reactions studied are quantitative. Confirmation of this conclusion was partly achieved by preliminary sequence analysis where at least 99% and 95% of the N-terminal residues of the products of reaction of the aminopeptidase and the hormones were found to be Phe and Asp for the human and bovine growth hormone products, respectively. Confirmation of the quantitative aspect of the reaction is confronted with obvious handicaps of the sequencing method (sensitivity, noise, by-products and separation limits) and the actual figures could be even higher than those given above.

It should be noted in this regard that the enzymatic reaction Met-Protein $\rightleftarrows$ Met + Protein is reversible and it could in principle be driven to synthesis by adding excess methionine or to complete hydrolysis by continuous removal of the amino acid. In one of the examples (Example V) demonstrating a batch preparation of hGH we have indeed employed ultrafiltration for several hours at a progressive stage of the reaction to remove free methionine and assist completion of the reaction.

Removal of the aminopeptidase from the reacted hormone is achieved by selective absorption and desorption of the hormone to an anion-exchange resin. Other alternative ways to

remove the aminopeptidase after its reaction with the hormone could be the use of a water-insoluble derivative of the enzyme in a batch or packed in a column as well as the use of an affinity resin for the enzyme to absorb it at the end of the reaction.

The procedure used for Met-hGH, Met-Asp-Gln-bGH, Met-Leu-hGH, Met-gamma-interferon, Met-somatomedin C, Met-pGH, Met-apolipoprotein E, and Met-bGH should be applicable to other growth hormones and polypeptides. Methods for obtaining the aminopeptidase can be improved (e.g. more economical process of isolation or genetically engineering the enzyme or developing microorganism overproducing aminopeptidase and endopeptidase-free mutants of the microorganism). Other aminopeptidases of low molecular weight (less than 100,000) like Streptomyces griseus aminopeptidase and aminopeptidases which are themostable and active at alkaline pH could possibly substitute for the Aeromonas enzyme.

In addition to its action on growth hormones the aminopeptidase(s) can be useful for other recombinant DNA products such as hormones, growth factors, and enzymes that possess N-terminal sequences in accordance with the specificity of the enzyme or enzymes, e.g. somatomedins interleukin 3, interferons, apolipoprotein E. Furthermore, the recombinant DNA products can be designed in a manner which would allow the removal of several amino acids from the N-terminus, in addition to the methionine residue. For example derivatives like Met-Lys-bGH, Met-Leu-Tyr-bGH and Met-Phe-Asp-Gln-bGH when acted upon by aminopeptidase will yield hGH, bGH, bGH and Asp-Gln-bGH, respectively. It may be also possible to use the enzyme to add an amino acid by using excess of the amino acid in the incubation mixture, thus driving the synthesis reaction.

What is claimed is:

1. A method of sequentially removing N-terminal amino acid residues from an analog of a eucaryotic polypeptide synthesized in a foreign host which comprises contacting the eucaryotic polypeptide analog with an appropriate aminopeptidase under suitable conditions permitting sequential removal of N-terminal amino acid residues, the polypeptide analog containing an amino acid residue or sequence of residues located at a position other than the N-terminus of the polypeptide analog which stops the action of the aminopeptidase.

2. A method of claim 1, wherein the foreign host is a bacterium.

3. A method of claim 1, wherein the aminopeptidase is stable at a temperature up to about 65°C.

4. A method of claim 1, wherein the aminopeptidase is stable and active at a pH of about 7.0.

5. A method of claim 1, wherein the aminopeptidase is stable and active at a pH from about 8.0 to about 10.0.

6. A method of claim 1, wherein the aminopeptidase has a molecular weight less than about 100,000.

7. A method of claim 6, wherein the aminopeptidase is of bacterial origin.

8. A method of claim 7, wherein the aminopeptidase is extracellular.

9. A method of claim 1, wherein the aminopeptidase is insoluble in water.

10. A method of claim 1, wherein the aminopeptidase is bound to a solid support.

11. A method of claim 1, wherein suitable conditions comprise adding an affinity resin to remove excess aminopeptidase.

12. A method of claim 7, wherein the aminopeptidase is Aeromonas aminopeptidase.

13. A method of claim 7, wherein the aminopeptidase is Streptomyces griseus aminopeptidase.

14. A method of claim 7, wherein the aminopeptidase is Bacillus stearothermophilus aminopeptidase II or III.

15. A method of claim 1, wherein the eucaryotic polypeptide analog is an analog of a hormone.

16. A method of claim 1, wherein the eucaryotic polypeptide is an analog of a lymphokine.

17. A method of claim 1, wherein the eucaryotic polypeptide analog is an analog of a growth factor.

18. A method of claim 1, wherein the eucaryotic polypeptide analog is an anolog of interferon.

19. A method of claim 1, wherein the eucaryotic polypeptide analog is an analog of somatomedin.

20. A method of claim 1, wherein the eucaryotic polypeptide analog is an analog of apolipoprotein E.

21. A method of claim 15, wherein the hormone is an growth hormone.

22. A method of claim 18, wherein the growth hormone is human growth hormone.

23. A method of claim 18, wherein the growth hormone is bovine growth hormone.

24. A method of claim 18, wherein the growth hormone is porcine growth hormone.

25. A method of claim 1, wherein the N-terminal amino acid residue is methionine.

26. A method of claim 21, wherein the amino acid residue or sequence of residues which stops the action of the aminopeptidase is located adjacent the N-terminal methionine.

27. A method of claim 21, wherein the amino acid residue or sequence of residues which stops the action of the aminopeptidase is separated from the N-terminal methionine by one or more amino acid residues.

28. A method of claim 12, wherein the amino acid residue which stops the action of the aminopeptidase is aspartic acid.

29. A method of claim 12, wherein the amino acid residue which stops the action of the aminopeptidase is glutamic acid.

30. A method of claim 12, wherein the amino acid sequence of residues which stops the action of the aminopeptidase comprises an amino acid residue other than proline bound to the N-terminus of a proline residue.

31. A method of claim 26, wherein the amino acid residue is phenylalanine.

32. A method of claim 1, wherein the eucaryotic polypeptide analog has the sequence Met-Phe-Pro- at its N-terminus.

33. A method of claim 18, wherein the growth hormone analog has the amino acid methionine as its N-terminus.

34. A method of claim 20, wherein the bovine growth hormone analog has the sequence Met-Asp-Gln- at its N-terminus.

35. A method of claim 20, wherein the bovine growth hormone analog has the amino acid methionine as its N-terminus.

36. A method of removing the N-terminal methionine residue from an animal growth hormone analog produced in bacteria by expression of a gene encoding the hormone which comprises contacting the growth hormone analog with Aeromonas aminopeptidase under suitable conditions permitting removal of the N-terminal methionine residue.

37. A method of removing the N-terminal methionine residue from a human growth hormone analog produced in bacteria by expression of a gene encoding the hormone, the human growth hormone analog having a methionine residue added to the N-terminus of authentic human growth hormone, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

38. A method of removing the N-terminal methionine residue from a bovine growth hormone analog produced in bacteria by expression of a gene encoding the bovine growth hormone analog, the bovine growth hormone analog having a methionine residue added to its N-terminus, which comprises contacting the analog with Aeromonas aminopeptidase under suitable conditions permitting the removal of the N-terminal methionine residue.

39. A method of adding an N-terminal amino acid residue to a polypeptide molecule which comprises contacting the polypeptide molecule with an aminopeptidase and a sufficient excess of the free amino acid residue to be added under suitable conditions permitting addition of the amino acid to the N-terminus of the polypeptide.

40. A method as in claim 35, wherein the aminopeptidase is Aeromonas aminopeptidase.

41. A method of claim 12, wherein the Aeromonas aminopeptidase is hyperactivated by metal substitutions.

42. A method of claim 36, wherein the metal substitutions comprise partially substituting Cu(II) for Zn(II) as a coenzyme.

43. A method as in claim 36, wherein the metal substitutions comprise partially substituting Ni(II) for Zn(II) as a coenzyme.

44. A polypeptide analog produced by the method of claim 1.

45. An growth hormone analog produced by the method of claim 18.

46. A human growth hormone analog produced by the method of claim 19.

47. Human growth hormone produced by the method of claim 19.

48. A bovine growth hormone analog produced by the method of claim 20.

49. A bovine growth hormone analog produced by the method of claim 30.

50. Bovine growth hormone produced by the method of claim 31.

51. The bovine growth hormone analog Asp-Gln-bGH.

52. A method of preparing an analog of a eucaryotic polypeptide which comprises:

a) producing a first analog in bacteria by expression of gene encoding the analog of the eucaryotic polypeptide;

b) removing the N-terminal methionine residue by the method of claim 1; and

c) recovering the resulting analog.

53. A method of preparing an analog of a eucaryotic polypeptide which comprises:

a) producing a first analog in bacteria by expression of a gene encoding the eucaryotic polypeptide;

b) removing the N-terminal methionine residue by the method of claim 12; and

c) recovering the resulting analog.

54. A method of claim 48, wherein the analog of a eucaryotic polypeptide is an analog of a growth hormone.

55. A method of claim 49, wherein the analog of a eucaryotic polypeptide is an analog of human growth hormone.

56. A method of claim 49, wherein the analog of a eucaryotic polypeptide is an analog of bovine growth hormone.

57. A method of claim 48, wherein the removing of the N-terminal is optimized by removing the free methionine residue by dialysis.

58. An analog of a growth hormone produced by the method of claim 50.

59. An analog of human growth hormone produced by the method of claim 51.

60. An analog of bovine growth hormone produced by the method of claim 52.

FIG. 1

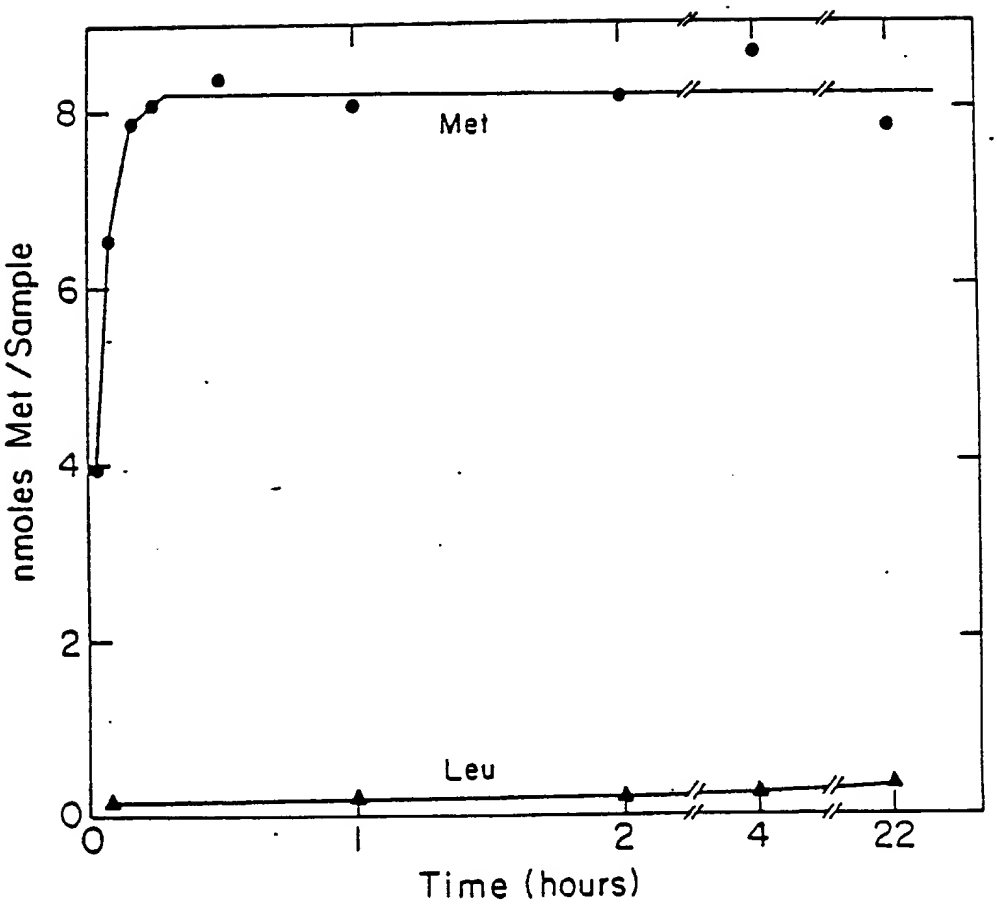
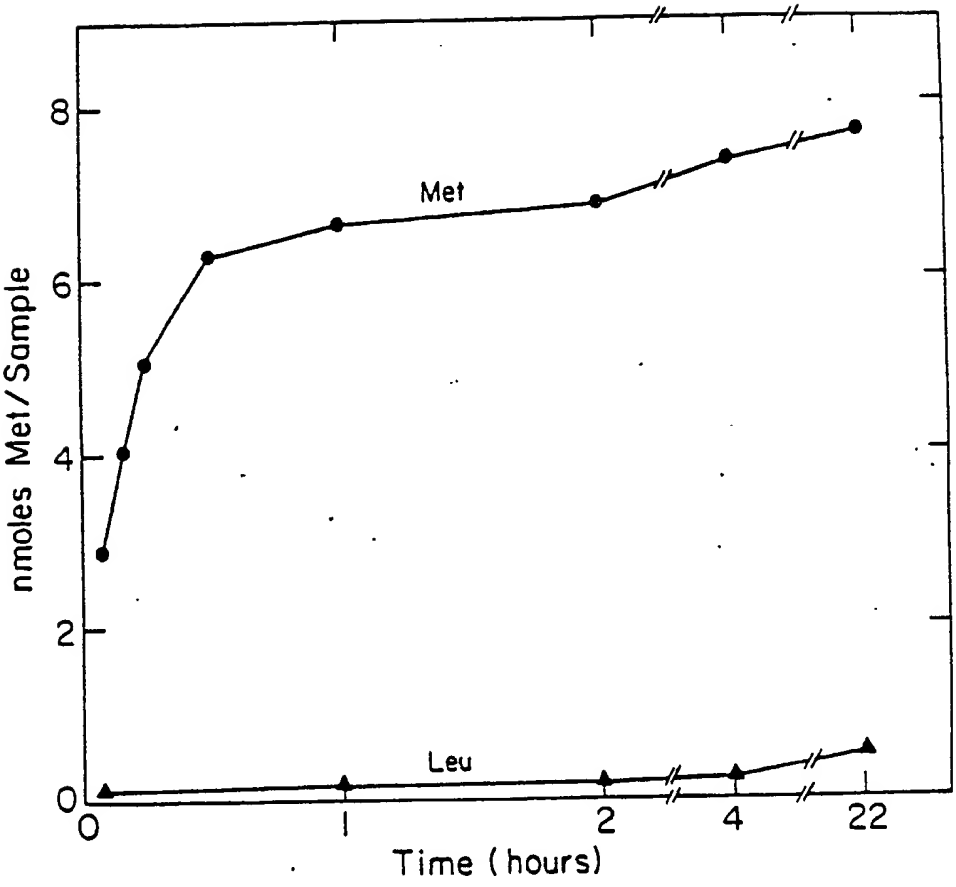


FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01531

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

C12P 21/00
435/68

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68, 69, 70, 172.3, 212, 219, 220, 253, 317, 832, 897; 935/47, 51 260/112R and 112.5R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

CHEMICAL ABSTRACTS DATA BASE -1971-1985 BIOSIS DATA BASE 1969-1985

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	EP, 20,290, Published 10 December 1980, Schering AG	1,2,15,19, 21, 25,26,52
Y	EP, 20,290, Published 10 December 1980 Schering AG	3-14,16-18, 20,27-33,36-43,53,56
X	WO 84/02351, Published 21 June 1984, Nordisk Insulin Laboratorium	1,2,7,14,15, 19,21, 25,26 52
Y	WO 84/02351, Published 21 June 1984, Nordisk Insulin Laboratorium	3-6, 8-13,16-20,27-33,36-43, 53, 56

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
30 October 1985	01 NOV 1985
International Searching Authority [1]	**Signature of Authorized Officer** [20]
RO/US	J. Hulea[illegible] Jayme A Hulcatt

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE FIRST SHEET
(Not for publication)

I. CERTAIN CLAIMS FOUND UNSEARCHABLE

Claims 22-24, 34, 35, 54, 55, and 57.

The above claims are improper dependent claims because they do not further limit the claims and are confusing, ambiguous and indefinite.

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [ ] Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. [X] Claim numbers *, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

* 22-24, 34, 35, 54, 55 and 57

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-43 and 52-57 are drawn to a method of producing a polypeptide classified in 435/68.

II. Claims 44-51 and 58-60 are drawn to polypeptides, particu- growth hormones and analogs there of classified in 260/112R.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.